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ORIGINAL CONTRIBUTION

Prospective study of telomere length and LINE-1 methylation in peripheral blood cells: the role of B vitamins supplementation

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Abstract
Purpose Deficiencies of folate, vitamins B_{12} and D are common age-related conditions. Vitamin B_{12} and folate are necessary for DNA methylation. Telomeres appear to be regulated by DNA methylation. Here, we study the effect of B vitamins supplementation on telomere length and global DNA methylation in a prospective study.
Methods In total, 60 elderly subjects were supplemented for 1 year with either vitamin B_{12}, B_6, folate, vitamin D and calcium (group A $n = 31$) or only vitamin D and calcium (group B $n = 29$). LINE-1 methylation, relative telomere length (T/S), vitamin B_{12}, folate, homocysteine (tHcy), 5-methyltetrahydrofolate (5-methylTHF), *S*-adenosylhomocysteine (SAH), *S*-adenosylmethionine (SAM), cystathionine and vitamin D were quantified before and after supplementation.
Results At baseline, tHcy was high, vitamin D was low, and T/S did not differ between groups A and B. Vitamin supplementation increased LINE-1 methylation in group A at site 317 but reduced LINE-1 methylation in group B at site 327. There was no correlation between T/S and LINE-1 methylation at baseline. Multiple backward regression analysis revealed baseline tHcy and 5-methylTHF are significant predictors of T/S. After supplementation in group B but not in group A, LINE-1 methylation correlated inversely with T/S, and LINE-1 methylation variation was an independent predictor of T/S variation. B vitamins decreased tHcy significantly in group A. Multiple backward regression analysis showed 5-methylTHF in group A and tHcy in group B were significant predictors for LINE-1 methylation. At baseline, the lower LINE-1 methylation observed in subjects with 5-methylTHF >10 nmol/l was in agreement with a reduced methyl group transfer due to a lower SAM formation. In group B, an increase in telomere length was correlated with lower LINE-1 methylation. Subjects with hyperhomocysteinemia >12 µmol/L had compared to those with normal tHcy a reduced LINE-1 methylation accompanied by a higher SAM and SAH (that inhibits demethylation of SAM) as well as lower 5-methylTHF. Additionally, subjects with tHcy > 12 µmol/L had longer telomeres when compared with subjects having tHcy < 12 µmol/L.
Conclusions The results suggest a possible effect of B vitamins for telomere biology in blood cells. Suboptimal B vitamins status and hyperhomocysteinemia are associated with altered DNA methylation and telomere length. These data have to be confirmed in future studies.

Keywords B vitamins · Telomere length · DNA methylation

Introduction

Folate, vitamin B_{12} and D deficiencies are common in elderly individuals [1–3]. Emerging evidence suggests that low blood concentrations of these vitamins increase the

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risk of age-related diseases, such as cardiovascular disease, cognitive dysfunction, dementia and osteoporosis [1, 2]. At present, it is insufficiently understood how vitamin deficiencies contribute to the development of these age-related diseases.

Vitamin B_{12} and folate are important for DNA methylation and nucleotide biosynthesis [4]. DNA methylation is important for the regulation of gene expression, the maintenance of genomic stability and telomere length [5]. DNA hypomethylation instead is associated with reduced genomic stability and altered gene expression [6]. Not surprisingly, folate and vitamin B_{12} deficiencies can lead to inadequate DNA methylation and genomic instability [5, 7].

A substantial proportion of DNA methylation sites are located in repetitive sequences, such as long-interspersed nuclear elements (LINEs) [8]. There are over 600,000 LINEs across the genome, representing approximately 17–25 % of all methylation sites [8]. Therefore, LINEs methylation is considered a surrogate marker for global genomic methylation [8].

Telomeres are DNA–protein structures that cap the ends of chromosomes [9]. They protect chromosomes from degradation, unwanted recombination–fusion and inappropriate activation of the DNA damage response system (DDR) [9, 10]. Due to the inability of the DNA polymerase to fully replicate the telomeric region, telomeres of somatic cells are shortened with every single division by 50–200 bp until they become critically short and lose their protective function [9–11]. Cells with critically short telomeres become either senescent or apoptotic [12]. Hematopoietic stem cells, germ cells and tumor cells can bypass this "end-replication problem" by the activation of telomerase [9, 10].

Telomere length is regarded as an indicator of biological age and has been studied in various age-associated diseases including cancer, cardiovascular diseases, diabetes type 2, Alzheimer's and Parkinson's disease [12–14]. However, telomere biology is complex and longer telomeres are not necessarily a sign of good health and appropriate cell function [15]. For example, abnormally long telomeres were found in peripheral blood cells of breast cancer patients with poor prognosis [15] and in subjects exposed to arsenic [16].

Telomere length alteration induced by DNA hypomethylation and impaired nucleotide synthesis is an appealing concept that could link folate and vitamin B_{12} deficiency to aging- and age-related diseases. However, cross-sectional studies investigating the relationship between B vitamins and telomere length yielded inconsistent results [17–20]. The present study aimed to explore the effect of B and/or D vitamins supplementation on telomere length and global DNA methylation in a prospective clinical trial.

Materials and methods

Study population

A total of 96 elderly subjects (mean age 68.25 ± 10.12 years) from a coronary rehabilitation sport program were enrolled in this 1-year randomized double-blind supplementation study. Our department of Biometry and Epidemiology performed the randomization in groups A and B in a double-blind manner. Participants were divided into two groups. The first group (group A; 34 subjects) received 500 µg folic acid, 500 µg B_{12}, 50 mg B_6, 1200 IU vitamin D and 456 mg Ca daily for 12 months. The second group (group B; 31 subjects) was supplemented with 1200 IU vitamin D and 456 mg Ca daily for the same period. Sixty-five participants completed the study. Fasting blood samples were collected at baseline and after 1 year of supplementation. The study aimed originally to evaluate the effect of vitamin D and calcium carbonate (Ca) with or without B vitamins on bone markers [21]. The detailed study design including inclusion and exclusion criteria has been reported previously [21]. The study was approved by the local ethics committee of Saarland, and all participants gave their written informed consent. The trial was conducted in accordance with the Declaration of Helsinki.

LINE-1 methylation analysis

Genomic DNA from whole-blood samples was extracted using QIAamp DSP DNA Blood Mini Kit (Qiagen), according to the manufacturer's protocol. DNA was available from 60 subjects (group A $n = 31$; group B $n = 29$). LINE-1 methylation was performed by pyrosequencing using the PSQ 96 MA system (Qiagen) as reported previously [22, 23]. Briefly, DNA was initially treated with bisulfite and LINE-1 repetitive elements were amplified by PCR. PCR was performed in non-stringent conditions allowing the amplification of a pool of several thousand repeats. The following PCR primers were used: forward, 5′-GGGACACCGCTGATCGTTTATCTCACTAAAAAATACCAAACAA-3′, and reverse, 5′-TTTTTTGAGTTAGGTGTGGG-3′. The reverse primer and a universal biotinylated primer (5′-GGGACACCGCTGATGTTTA) were used for a nested PCR. The sequencing reaction was performed with two primers (5′-GGGTGGGAGTGAT-3′ and 5′-AGTTAGGTGTGGGATATAGT-3′). A sequence containing four tandem CpG sites (305, 317, 320 and 327 according to GenBank accession number X58075) was analyzed. The C (methylated) to T (unmethylated) ratio was calculated for each site by pyrosequencing method. Results are expressed as percentage of methylated cytosine (C) versus non-methylated cytosine (T). Mean

LINE-1 methylation was calculated as average of methylation at the four analyzed sites and was used as a surrogate marker for whole-genome methylation [22]. The relative change of LINE-1 methylation was calculated with the following equation [100 × ((study-end − baseline)/baseline)].

Telomere length analysis

Telomere length was measured in genomic DNA from whole blood using a real-time PCR-based assay, as previously reported [24, 25]. Briefly, the mastermix for each 20 µl PCR was prepared with 4 µl LightCycler Fast Start DNA Master Plus SYBR Green I (Roche), 150 nmol/L of telomere-specific primers: forward: 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′; reverse: 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′; or 100 nmol/L 36b4 (single-copy housekeeping gene) primers: forward: 5′-CAGCAAGTGGGAAGGTGTAATCC-3′; reverse: 5′-CCCATTCTATCATCAACGGGTACAA-3′ [24, 25]. In each run, 40 ng of sample DNA was measured in duplicate, a maximal coefficient of variation (CV) between replicates of 2.5 % was considered acceptable, and the average of both replicates was calculated. None of the samples in the study had to be excluded because of too much variance in both the original measurement and an eventual repeat measurement. The thermal cycling profile for both reactions consisted of a 95 °C activation step, followed by 40 cycles of 95 °C for 15 s and 58 °C for 60 s. All real-time PCRs were carried out on a LightCycler Instrument (Roche). A seven-point standard curve (dilution from 5 to 100 ng) was run using a pool of ten control DNAs for both the telomere and 36b4 PCRs to ensure linearity of the reaction ($R^2 > 0.99$) during optimization of the assays. PCR efficiencies for target and reference gene were approximately equal, 2.1 and 1.94, respectively. The pooled control DNA was tested in all assays to allow comparability of the results.

The PCR data were analyzed with the comparative C_t method $\left(2^{-\Delta\Delta C_t}\right)$ to calculate T/S ratio [24, 25]. Briefly, after calibration, the C_t of the target gene was subtracted by the C_t of the reference gene to calculate ΔC_t. The ΔC_t of the sample was subtracted by the ΔC_t of the control DNA to calculate the $\Delta\Delta C_t$. Finally, the T/S ratio was calculated using the $2^{-\Delta\Delta C_t}$ equation. This method measures the relative expression ratio of the target gene (telomere, T) in comparison with a reference gene (36b4, S). The relative change of T/S was calculated with the following equation [100 × ((study-end − baseline)/baseline)].

Measurement of serum markers

Fasting venous blood samples were collected in serum or EDTA tubes. Serum samples were allowed to clot for 30 min at room temperature before centrifugation for 10 min at 2000*g* and 4 °C. Plasma samples were spun within 30 min after collection for 10 min at 2000*g* and 4 °C. Serum and plasma were separated from blood cells and divided into several aliquots. For the measurement of S-adenosylhomocysteine (SAH) and *S*-adenosylmethionine (SAM), 500 µL EDTA plasma was acidified with 50 µL 1 M acetic acid and mixed thoroughly. All samples were stored at −70 °C until analysis.

Total folate and 5-methyltetrahydrofolate (5-methyl-THF) in serum were quantified using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), as recently described by Kirsch et al. [26, 27]. The concentrations of homocysteine (tHcy) and cystathionine were determined by a modified gas chromatography mass spectrometry method as described elsewhere [21, 28]. Total vitamin B_{12} was analyzed using an automated chemiluminescence immunoassay from Abbott Diagnostics (Abbott, Diagnostics, Delkenheim, Germany). SAH and SAM were all quantified by UPLC-MS/MS as previously described [22, 29]. 25-hydroxy vitamin D (25(OH)D) was measured with a commercial chemiluminescence immunoassay on a Liaison automated analyzer (DiaSorin, Saluggia, Italy) [21].

Statistical analysis

Statistical analyses were performed with SPSS (Statistical Package for the Social Sciences, version 21.0, SPSS Inc., Chicago, IL, USA).

All parameters were checked for normality, and where needed variables were log-transformed. The differences between independent variables were tested by Student's *t* test or Mann–Whitney *U* test (continuous variables) and by McNemar test (categorical variables). The differences between dependent variables were tested by the paired t test or the Wilcoxon test. Correlation analyses were performed adjusting for age and gender. A stepwise regression with backward variable selection was performed to construct models that predict T/S or mean LINE-1 methylation. The *p* value for inclusion in the backward regression model was set at 0.05 and for removal 0.1. All tests were two-sided. A *p* value <0.05 was considered statistically significant.

Results

The baseline characteristics of participants in groups A and B are shown in Table 1. In both groups, the median baseline concentration of tHcy was above the cutoff >12 µmol/L (Table 2). Hyperhomocysteinemia in subjects with normal renal function indicates B vitamin deficiency, and tHcy was normalized by B vitamins supplementation

Table 1 Characteristics of study participants, according to supplementation arm

Variable	Group A $n = 31$	Group B $n = 29$	p value
Females (%)	67.7 %	44.8 %	0.08
Age (year)	66 ± 10.55	70.62 ± 9.2	0.08
BMI (kg/m^2)	26.18 ± 2.88	26.9 ± 4.44	0.5
Diseases (%)[a]	42.9 %	44.4 %	0.9
Smoking	8.3 %	4.3 %	0.6
Alcohol consumption	70.8 %	50 %	0.2

Tie data are mean ± SD or percentage

[a] Diseases induced are: cardiovascular diseases, diabetes, cancer, hypertension

in group A ($p < 0.001$), but not in group B (without B vitamins supplementation) where it tended to increase ($p = 0.07$). After 1 year of supplementation, cross-sectional comparison between the two groups showed lower vitamin B_{12} ($p < 0.001$) and total folate ($p < 0.001$) and higher tHcy ($p < 0.001$) concentrations in group B (Table 2).

Telomere length at baseline and after 1 year of supplementation

At baseline, relative telomere length (T/S) in peripheral blood leukocytes ranged from 0.81 to 1.89 in group A and from 0.62 to 2.14 in group B (mean 1.26 ± 0.52; Table 2). After 1 year of supplementation, the T/S ratio ranged from 0.63 to 2.04 (mean 1.21 ± 0.53) in group A and from 0.61 to 2.23 (mean 1.3 ± 0.67) in group B. Neither at baseline nor at study end, the T/S was significantly different between groups A and B. Furthermore, there were no significant changes in average T/S within the individual groups.

Variation of LINE-1 methylation during 1 year of supplementation

LINE-1 methylation at baseline and after 1 year of supplementation is shown in Table 3. At baseline, mean LINE-1 methylation over all sites was slightly higher in group B than in group A. Furthermore, LINE-1 methylation at site 317 in group A and at site 327 in group B differed significantly between baseline and supplementation. Moreover, at the end of the study, LINE-1 methylation at site 305 differed significantly between groups A and B.

The relative change of LINE-1 methylation [100 × ((study-end − baseline)/baseline)] in the two groups during the 1-year supplementation period is illustrated in Fig. 1. In group B, supplementation resulted in a reduction of LINE-1 methylation at site 317 (mean percentage change in group A: +0.81 %; in group B: −0.59 %; $p = 0.04$) and at site 327 (mean percentage change in group A: +0.29 %; in group B: −1.50 %; $p = 0.04$).

Table 2 Changes of telomere length and vitamins, during one year of supplementation

	Baseline		After 1 year	
	Group A	Group B	Group A B, D, Ca	Group B D, Ca
	$n = 31$	$n = 29$	$n = 31$	$n = 29$
T/S (ratio)	1.2 ± 0.4	1.26 ± 0.5	1.21 ± 0.5	1.3 ± 0.7
Vitamin B_{12} (pmol/l)	**317 ± 140**$^{(*2)}$	294 ± 88	**511 ± 178**$^{(*1);\,(*2)}$	**271 ± 71**$^{(*1)}$
tHcy (nmol/l)	**12 ± 4**$^{(*2)}$	14 ± 6	**9 ± 3**$^{(*1);\,(*2)}$	**15.5 ± 9**$^{(*1)}$
Total folate (nmol/l)	**21 ± 14**$^{(*2)}$	22 ± 16	**52 ± 18**$^{(*1);\,(*2)}$	**18 ± 10**$^{(*1)}$
5-MethylTHF (nmol/l)	**19.4 ± 12**$^{(*2)}$	20 ± 14	**48 ± 17**$^{(*1);\,(*1)}$	**16 ± 10**$^{(*1)}$
SAH (nmol/l)	18 ± 7	21 ± 10	21 ± 10	22 ± 14
SAM (nmol/l)	125 ± 35	126 ± 33	129 ± 38	119 ± 36
Cystathionine (nmol/l)	**454 ± 753**$^{(*2)}$	**338 ± 411**$^{(\#3)}$	**191 ± 107**$^{(*1);\,(*2)}$	**461 ± 587**$^{(*1);\,(\#3)}$
25(OH)D (nmol/l)	**42 ± 19**$^{(*2)}$	**40 ± 21**$^{(\#3)}$	**81 ± 23**$^{(\#1);\,(*2)}$	**66 ± 20**$^{(\#1);\,(\#3)}$

The data are mean ± SD

Statistical significant differences are in bold

* $p < 0.001$; # $p < 0.005$

[1] Between groups A and B after 1 year (cross-sectional companion)

[2] Between group A baseline versus after 1 year (longitudinal comparison)

[3] Between group B baseline versus after 1 year (longitudinal comparison)

Table 3 LINE-1 methylation (%) as percentage of methylated cytosine versus non-methylated cytosine at baseline and after 1 year of supplementation

	Baseline		After 1 year of supplementation	
	Group A	Group B	Group A	Group B
			B, D, Ca	D, Ca
LINE-1 methylation site 305 (%)	81.1 ± 0.6	82.3 ± 0.6	**81.43 ± 0.6**$^{(*2)}$	**83.29 ± 0.5**$^{(*2)}$
LINE-1 methylation site 317 (%)	**82.9 ± 0.5**$^{(*1,3)}$	**84.4 ± 0.3**$^{(*1)}$	**83.69 ± 0.3**$^{(*3)}$	84.06 ± 0.1
LINE-1 methylation site 320 (%)	81.38 ± 0.5	82.02 ± 0.4	81.08 ± 0.4	81.67 ± 0.5
LINE-1 methylation site 327 (%)	76.99 ± 0.6	**78.35 ± 0.5**$^{(*4)}$	76.88 ± 0.5	**77.19 ± 0.4**$^{(*4)}$
Mean LINE-1 methylation (%)	**80.74 ± 0.4**$^{(*1)}$	**81.64 ± 0.2**$^{(*1)}$	80.77 ± 0.3	81.50 ± 0.3

The data are mean ± SD

Statistical significant differences are in bold

* $p < 0.05$

[1] Between groups A and B at baseline (cross-sectional comparison)

[2] Between groups A and B after 1 year (cross-sectional comparison)

[3] Between group A baseline versus after 1 year

[4] Between group B baseline versus after 1 year (longitudinal comparison)

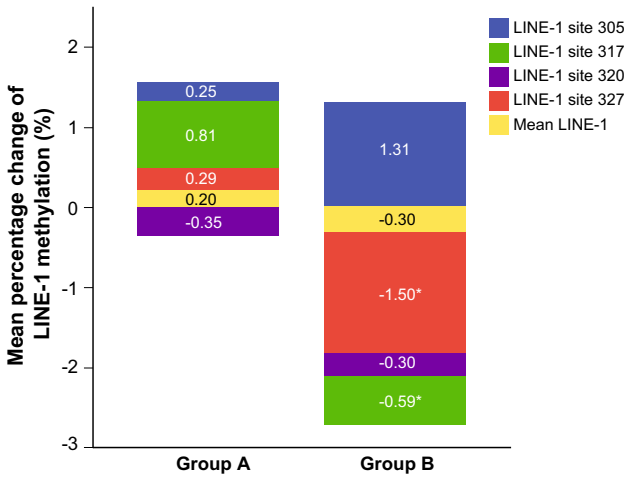

Fig. 1 Mean percentage change of LINE-1 methylation (%) from baseline to 1 year of supplementation in groups A and B. Data indicate the mean of percentage variation. *$p < 0.05$

Correlation between LINE-1 and telomere length

At baseline, there was no significant correlation between age- and gender-adjusted T/S and LINE-1 methylation at any site. After 1 year of supplementation, age- and gender-adjusted T/S percentage change was inversely correlated with mean LINE-1 methylation percentage change in group B ($r = -0.5$, $p = 0.008$), but not in group A ($r = -0.19$, $p = 0.33$), as reported in Fig. 2a. Similar correlations were also found at sites 317 ($r = -0.43$, $p = 0.028$, Fig. 2b) and 327 ($r = -0.41$, $p = 0.034$, Fig. 2c) in group B but not in group A (site 317 $r = -0.14$, $p = 0.48$; site 327 $r = 0.1$, $p = 0.61$). No correlations were found at sites 305 (group A $p = 0.4$; group B $p = 0.5$) and 320 (group A $p = 0.5$; group B $p = 0.08$).

LINE-1 methylation and telomere length variations

To better understand the relationship between telomere length and LINE-1 methylation, we analyzed separately subjects with an increase (positive T/S variation) or a decrease (negative T/S variation) in telomere length after 1 year of supplementation. Subjects of group B with an increase in T/S showed a significant reduction of mean LINE-1 methylation ($p = 0.001$) and LINE-1 methylation at site 327 ($p = 0.05$) when compared to those with a neutral or negative T/S variation (Fig. 3b).

Effect of homocysteine and 5-methylTHF on telomere length and LINE-1 methylation

Subjects with homocysteine concentrations >12 μmol/L had at baseline and after vitamin supplementation longer telomeres when compared with subjects having homocysteine <12 μmol/L ($p = 0.033$ and $p = 0.03$, respectively, Fig. 4). When the subjects were subdivided according to treatment arms, we found no significant difference in T/S between the group of subjects having tHcy< or >12 μmol/L (baseline group A $p = 0.4$, group B $p = 0.06$; after supplementation group A $p = 0.2$, group B $p = 0.1$).

At baseline, we found in subjects with high 5-methylTHF > 10 nmol/L compared with those having <10 nmol/L 5-methylTHF a lower LINE-1 methylation (Fig. 5a). Additionally, we found in this group lower SAH and SAM as

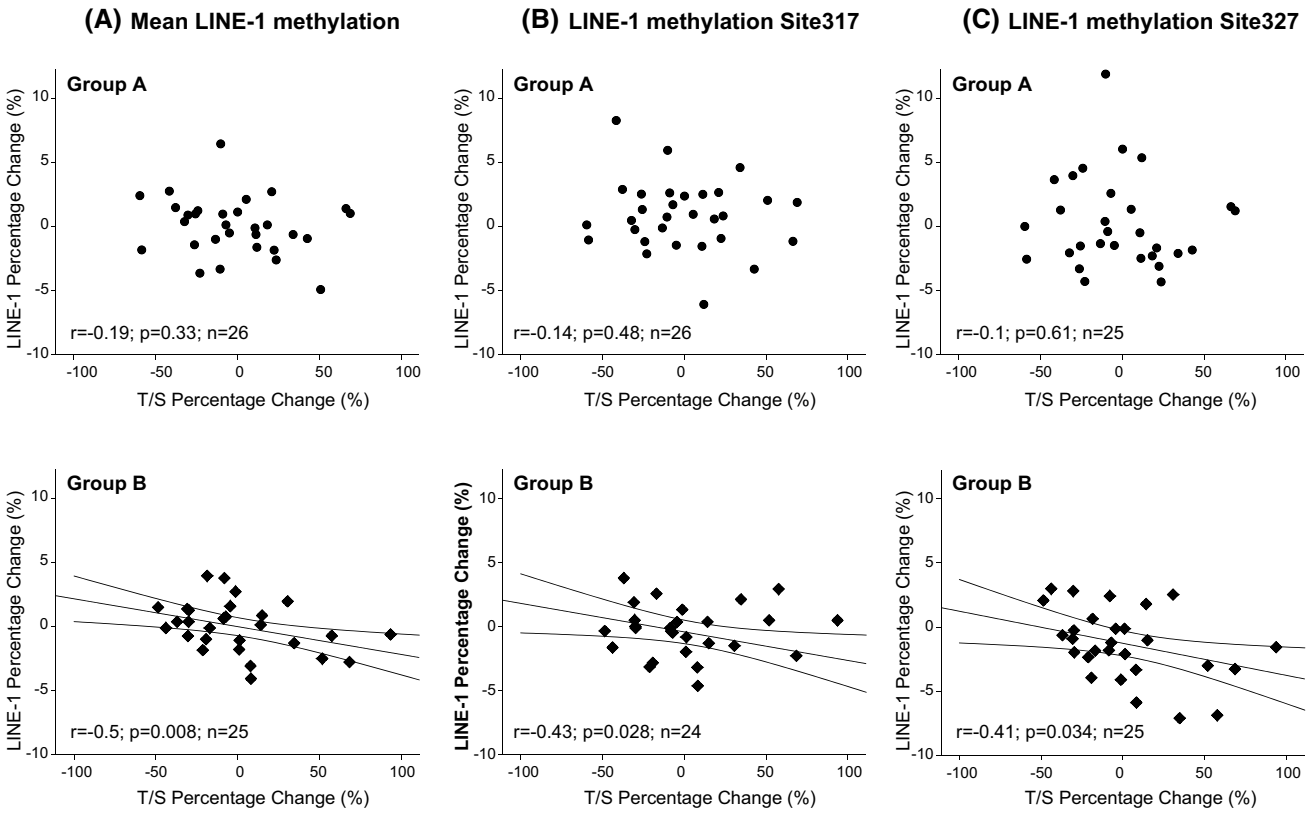

Fig. 2 Correlation of age- and gender-adjusted relative leukocyte telomere length (T/S) and LINE-1 methylation status at different sites. **a** Age- and sex-adjusted correlation between T/S percentage change (%) and mean LINE-1 methylation percentage change in group A (*top panel*) and group B (*bottom panel*). **b** Age- and sex-adjusted correlation between T/S percentage change (%) and LINE-1 methylation at site 317 percentage change in group A (*top panel*) and group B (*bottom panel*). **c** Age- and sex-adjusted correlation between T/S percentage change (%) and LINE-1 methylation at site 327 percentage change in group A (*top panel*) and group B (*bottom panel*)

well as significantly lower tHcy and cystathionine (Fig. 5b, c). In this group, the lower LINE-1 methylation is most probably in agreement with a reduced methyl group transfer due to a lower SAM formation.

Figure 5d shows that subjects with hyperhomocysteinemia >12 µmol/L have compared to the group with normal tHcy a reduced LINE-1 methylation which is accompanied by a higher SAH and SAM as well as significantly lower 5-methylTHF and higher cystathionine level (Fig. 5e, f). This metabolic picture is in agreement with an inhibited transmethylation because high tHcy leads to higher SAH and cystathionine. SAH inhibits the demethylation of SAM to SAH. This might explain the lower LINE-1 methylation in group B having tHcy > 12 µmol/L. Statistical analyses of subjects with high or low tHcy levels (cutoff 12 µmol/L) in group A after supplementation were not performed as only three subjects had tHcy above 12 µmol/L.

Multiple linear regression analyses

Multiple regression analysis revealed that at baseline, plasma tHcy as well as 5-methylTHF had an independent and significant effect on telomere length (Table 4a). Furthermore, after supplementation in group B, LINE-1 methylation variation at site 317 and site 327 had an independent and significant effect on telomere length variation (Table 4b). This was not observed in group A after supplementation.

In addition, 5-methylTHF ($p = 0.06$, Table 4c) in group A and tHcy ($p = 0.04$, Table 4d) in group B had an independent effect on mean LINE-1 methylation. Vitamin D was not a predictor for telomere length or LINE-1 methylation at baseline nor after supplementation.

Discussion

Information regarding baseline concentrations and after vitamins supplementation of telomere length, vitamins and metabolites in serum for each group is provided in Table 2. Vitamin B_{12} and folate increased in group A because of supplementation of both vitamins. In group B without supplementation with B vitamins, we did not observe such an increase; in contrast, we noticed a further decrease in

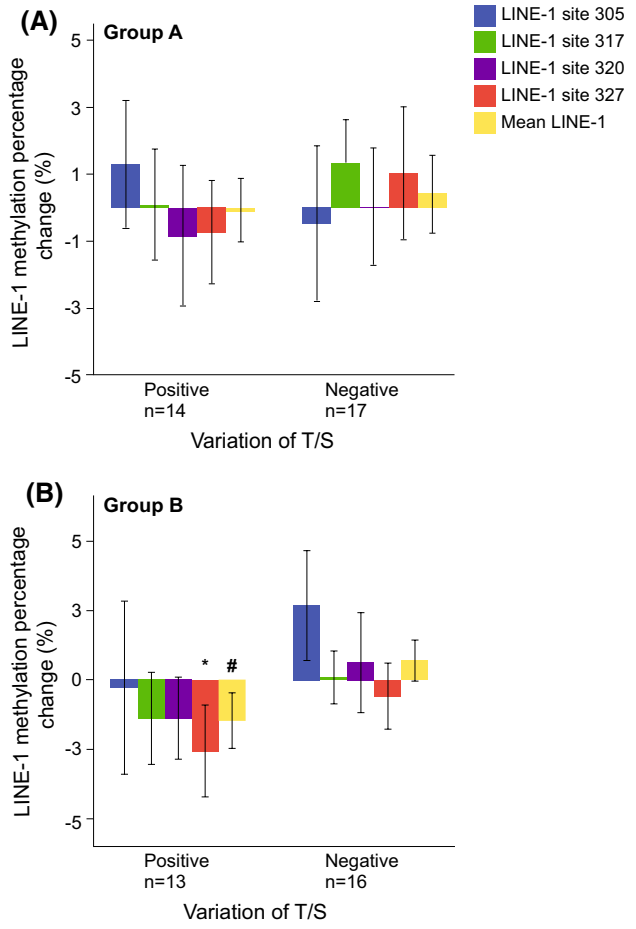

Fig. 3 LINE-1 methylation status according to telomere length variation. **a** Percentage change of LINE-1 methylation at sites 305, 317, 320, 327 and mean LINE-1 methylation according to T/S variation (negative or positive) in group A (group A positive variation $n = 14$, negative variation $n = 17$). **b** Percentage change of LINE-1 methylation at sites 305, 317, 320, 327 and mean LINE-1 methylation according to T/S variation (negative or positive) in group B (group B positive variation $n = 13$, negative variation $n = 16$). $^{*}p < 0.05$; $^{\#}p < 0.001$

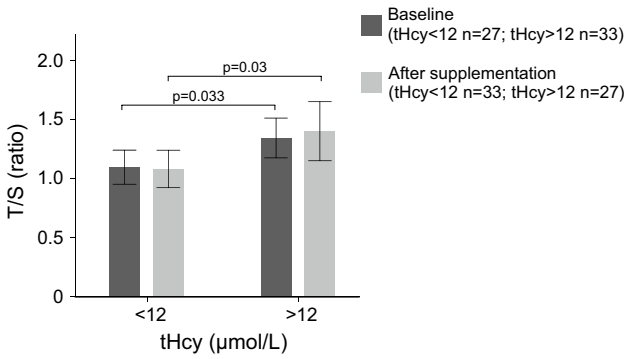

Fig. 4 T/S variation according to tHcy concentration (<12 µmol/L or >12 µmol/L) at baseline and after supplementation. Baseline tHcy < 12 µmol/L $n = 27$, tHcy > 12 µmol/L $n = 33$; after supplementation, tHcy < 12 µmol/L $n = 33$, tHcy > 12 µmol/L $n = 27$

folate and vitamin B_{12} serum concentrations. Moreover, we observed an increase in vitamin D in both groups after supplementation, because vitamin D was supplemented in both groups. The increase in vitamin D was higher in group A than in group B. A possible explanation for the somewhat lower increase in vitamin D in group B is most probably due differences in dietary habits and the higher age in this group. Furthermore, PTH (not reported), which is controlled by vitamin D, was in group B also higher (73 ng/ml) than in group A (67 ng/ml) but after supplementation PTH dropped to same level (46 pg/ml) in both groups.

The present study provides longitudinal evidence for a dynamic relationship between telomere length, LINE-1 methylation and B vitamin status. Individuals with a suboptimal vitamin B status showed altered DNA methylation and telomere elongation.

Our results are in agreement with previous studies suggesting that telomere length is a dynamic feature [30]. In a longitudinal study, Weischer et al. [31] measured telomere length in leukocytes of over 4000 subjects at baseline and again after 10 years of follow-up. The change in telomere length was heterogeneous, 56 % of their study cohort lost, and 44 % gained telomere length during these 10 years. Similar findings were reported by others [15, 32–36]. For example, Aviv et al. [35] showed a highly variable rate of change in leukocyte telomere length (increases and losses) during 12 months of observation. In line with these findings, our results support the notion that telomere length in leukocytes might fluctuate during life time [36]. Indeed, telomere length at a defined time point is the result of potential regulatory mechanisms and environmental factors [36]. In our study, we found that B vitamin status is a significant environmental factor influencing DNA methylation and telomere length. The change of telomere length over time is a multifactorial process that depends, at least partially, on DNA methylation.

In group B, where individuals were not supplemented with B vitamins, mean LINE-1 methylation was negatively associated with peripheral blood cell telomere length. This association was confirmed by the multiple linear regression analysis. At study end, group B was characterized by lower serum vitamin B_{12} and high tHcy when compared to group A. Group A was supplemented with B and D vitamins so that subjects were saturated with these vitamins. Not surprisingly, serum concentrations of vitamins B_{12} and D were within the respective reference range in all subjects of group A. Furthermore, the dispersion of tHcy was significantly less in group A than in group B suggesting an appropriate correction of vitamin stores. Considering the saturation of vitamin stores in group A, the lack of correlation between telomere length and DNA methylation is to be expected and does not contrast a functional relationship between DNA methylation and telomere length.

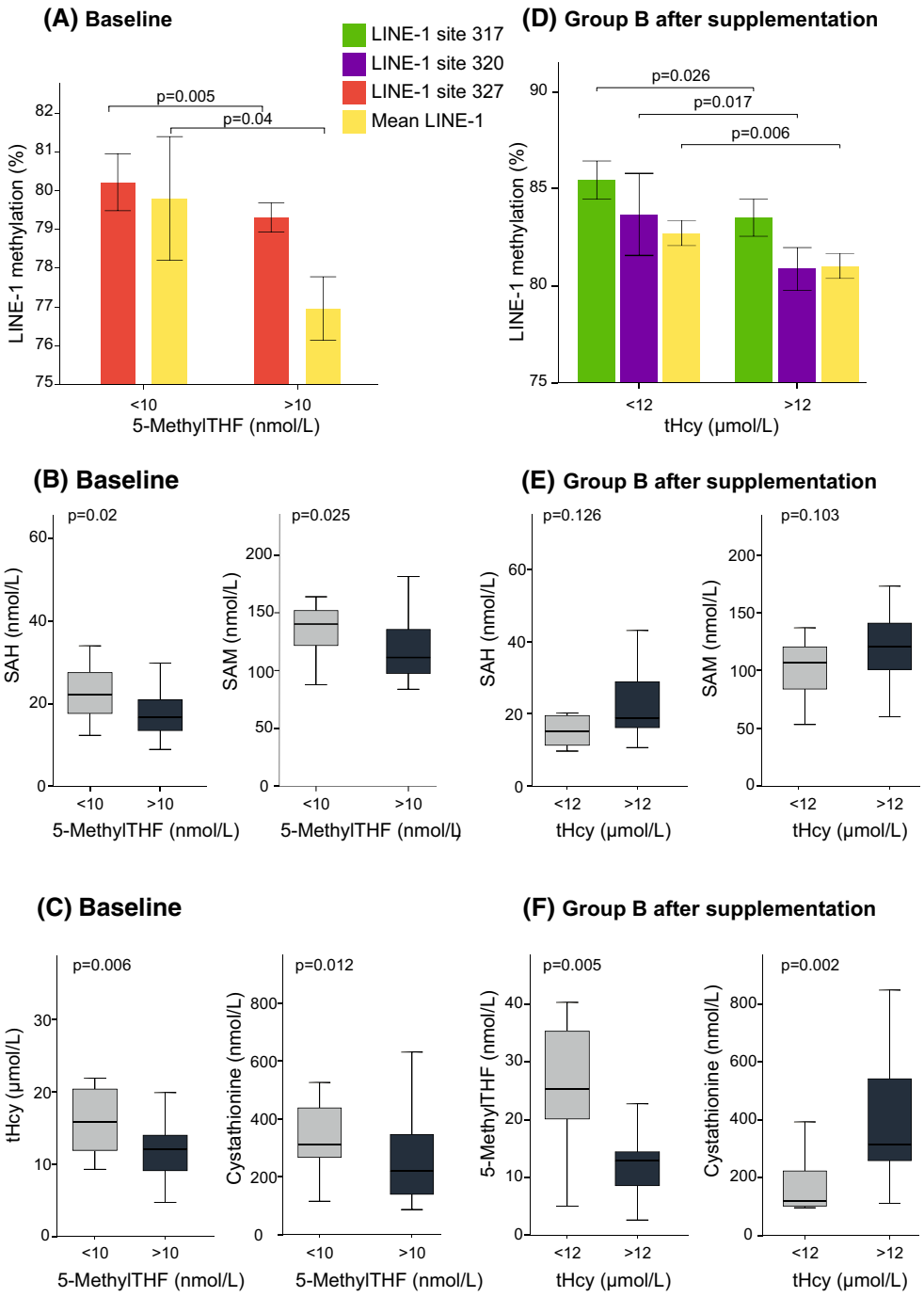

Fig. 5 **a** Mean LINE-1 methylation and LINE-1 methylation at site 327 according to 5-methylTHF status (<10 nmol/L $n = 14$ or >10 nmol/L $n = 46$), at baseline. **b** SAH and SAM according to 5-methylTHF status (<10 nmol/L $n = 14$ or >10 nmol/L $n = 46$), at baseline. **c** tHcy and cystathionine according to 5-methylTHF status (<10 nmol/L $n = 14$ or >10 nmol/L $n = 46$), at baseline. **d** Mean LINE-1 methylation and LINE-1 methylation at sites 317 and 320 according to tHcy concentration (<12 µmol/L $n = 12$ or >12 µmol/L $n = 17$), after supplementation in group B. **e** SAH and SAM according to tHcy concentration (<12 µmol/L $n = 12$ or >12 µmol/L $n = 17$), after supplementation in group B. **f** 5-methylTHF and cystathionine according to tHcy concentration (<12 µmol/L $n = 12$ or >12 µmol/L $n = 17$), after supplementation in group B

So far, only a few studies have explored the relationship between telomere length and B vitamin status. The results of these studies are conflicting. For example, in a population-based cohort of 1207 subjects (91.5 % women), low levels of plasma folate and high levels of tHcy were associated with shorter telomeres [17]. Other studies were not able to confirm this result [18–20]. In the Framingham Offspring Cohort Study (1044 subjects), a negative relationship between plasma folate concentration and telomere length was observed [20]. The findings of our prospective study showed that subjects with high tHcy > 12 µmol/L had longer telomeres compared with those having plasma tHcy < 12 µmol/L. However, most previous studies harbor significant limitations. They were mainly of cross-sectional nature, and in one large study, B vitamin status was assessed only by questionnaire [19]. Therefore, it remains unclear whether folate and vitamin B_{12} are related to telomere length and telomere biology.

As expected, mean telomere length of the groups did not change within the short period of 1 year. However,

Table 4 Summary of the stepwise regression analyses with backward variable selection

Dependent variable	Independent variable	Estimated effect	Two-sided *p* value
(A) T/S at baseline[a]	5-MethylTHF	0.584	0.003
	tHcy	0.540	0.006
(B) T/S percentage change in group B (Vit. D and Ca) after supplementation[b]	LINE-1 site 317 percentage change	−0.388	0.027
	LINE-1 site 327 percentage change	−0.385	0.028
(C) Mean LINE-1 in group A (Vit. B, Vit. D, Ca) after supplementation[c]	5-MethylTHF	−0.398	0.06
(D) Mean LINE-1 in group B (Vit. D, Ca) after supplementation[c]	tHcy	−0.68	0.004

[a] Variables included in the analysis were as follows: 5-methylTHF, tHcy, vitamin B_{12}, vitamin D, mean LINE-1 methylation and LINE-1 methylation at sites 305, 317, 320, 327

[b] Variables included in the analysis were as follows: percentage change of LINE-1 methylation at sites 305, 317, 320, 327 and mean LINE-1

[c] Variables included in the analysis were as follows: 5-methylTHF, tHcy, vitamin B_{12} and vitamin D

when individuals are looked at prospectively (a strength of this study), subtle differences are found which interestingly correlate with a global marker of DNA methylation. In fact, subjects of group B with an increase in telomere length during supplementation showed a reduction of mean LINE-1 methylation. This was not seen in individuals with a reduction of telomere length and suggests an association between global DNA hypomethylation and elongated telomeres. A functional relationship between DNA hypomethylation and elongated telomeres is also supported by an in vitro study using DNA methyltransferase (DNMT)-deficient cells. In this study, Gonzalo et al. [37] showed that loss of DNA methylation occurs together with elongated telomeres. Further evidence for this concept comes from another cell culture study where cells were cultured for up to 28 days in folate-deficient or folate-repleted medium [38]. After 14 days of culture, they found 26 % longer telomeres in cells cultured in folate-deficient medium when compared to cells cultured in folate-repleted medium. This was followed by rapid telomere attrition over the subsequent 14 days. Both long and short telomeres were positively correlated with biomarkers of chromosome instability, including micronuclei, nucleoplasmic bridges, nuclear buds and fused nuclei [38]. The early increase in telomere length in folate-deprived cells was associated with global DNA hypomethylation [38]. However, in the absence of other cell culture studies, it remains unclear whether these results are reproducible and apply to other cell types as well. The multiple regression analysis of our study supports that homocysteine and 5-methylTHF are independent predictors of telomere length and LINE-1 methylation. Furthermore, we found that tHcy > 12 µmol/L was accompanied with longer telomeres and 5-methylTHF <10 nmol/L was linked with significantly higher LINE-1 methylation (mean and at site 327) due to a lower SAM formation.

The association between telomere length and global DNA methylation was also investigated in a cohort of 84 boilermakers [39]. In this study, the rate of telomeric change was modified by the degree of LINE-1 methylation [39]. In contrast to our findings, global DNA hypomethylation was associated with shorter telomeres. The different results could be due to differences in the composition of the two study cohorts. While Wong et al. studied healthy males, our cohort included elderly subjects of both genders affected by a range of age-related diseases. Moreover, the two studies measured LINE-1 methylation at different sites. In another study, Li et al. [40] analyzed telomere length, DNA methylation and markers of chromosomal aberrations in peripheral blood in a cohort of healthy Norwegian men. They found a positive correlation between LINE-1 methylation and chromatid aberrations but not with telomere length [40].

Finally, it should be mentioned that telomeres and LINE-1 may also directly interact. Recently, Morrish et al. [41] proposed an interesting mechanism by which LINE-1 interacts with telomeres. LINE-1 can integrate at dysfunctional telomeres by a mechanism called alternative endonuclease-independent retrotransposition. This phenomenon was described in Chinese hamster ovary cell lines deficient for p53 and for factors that are important in the non-homologous end-joining pathway of DNA repair [41]. Retrotransposon can also bind to and elongate telomeres when they are uncapped. If this is an actual mechanism in humans that is not clear, but putatively, LINE-1–telomere interaction is an ancestral evolutionary mechanism which served to stabilize chromosomes and telomeres before telomerase evolved [41].

In summary, existing data regarding telomere length and global DNA methylation are inconclusive and require further investigation.

One major limitation of the present study is the small sample size. However, the number of subjects was sufficient to demonstrate a significant association between telomere length and DNA methylation and biomarkers

of methyl group availability like folate, SAM and tHcy. Another limitation of this study is the use of a qPCR-based method for the assessment of telomere length. This method measures only relative length, but does not provide an absolute telomere length. Moreover, it measures the mean telomere length of all chromosomes in a sample and not chromosome-specific telomere length. However, the qPCR-based method is sufficiently cost-effective to allow high-throughput telomere length analyses. In fact, qPCR-based methods are widely used in epidemiological studies. Finally, T/S ratio is only one indicator, and optimally telomerase activity and telomere shelterin proteins such as TRF2, which strongly regulate telomere length, were not measured due to the design of the study. With respect to the samples analyzed, it must be acknowledged that whole-blood DNA was used for the assay and contribution of certain cell populations cannot be distinguished.

In summary, the present trial suggests that B vitamin deficiency has an impact on telomere length, which is possibly mediated by altered DNA methylation. Considering the small study population and inconsistent results of previous studies, further work is needed to clarify this important aspect of cellular aging. In particular, large longitudinal observation studies and supplementation studies with a longer follow-up period and multiple prospective blood collections will help to shed further light on the role of epigenetics in telomere biology in the context of vitamin supplementation.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

1. Stabler SP (2013) Clinical practice. Vitamin B_{12} deficiency. N Engl J Med 368:149–160
2. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357:266–281
3. Herrmann W, Schorr H, Bodis M, Knapp JP, Muller A, Stein G, Geisel J (2000) Role of homocysteine, cystathionine and methylmalonic acid measurement for diagnosis of vitamin deficiency in high-aged subjects. Eur J Clin Invest 30:1083–1089
4. Hughes CF, Ward M, Hoey L, McNulty H (2013) Vitamin B_{12} and ageing: current issues and interaction with folate. Ann Clin Biochem 50:315–329
5. Moores CJ, Fenech M, O'Callaghan NJ (2014) Telomere dynamics: the influence of folate and DNA methylation. Ann N Y Acad Sci 1229:76–88
6. Wainfan E, Poirier LA (1992) Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. Cancer Res 52:2071s–2077s
7. Nazki FHSAGB (2014) Folate: metabolism, genes, polymorphisms and the associated diseases. Gene 533:11–20
8. Kazazian HH (2014) Mobile elements: drivers of genome evolution. Science 303:1626–1632
9. Blasco MA (2007) Telomere length, stem cells and aging. Nat Chem Biol 3:640–649
10. Blasco MA (2005) Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet 6:611–622
11. Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 37:614–636
12. Fyhrquist F, Saijonmaa O, Strandberg T (2013) The roles of senescence and telomere shortening in cardiovascular disease. Nat Rev Cardiol 10:274–283
13. Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA (2003) Association between telomere length in blood and mortality in people aged 60 years or older. Lancet 361:393–395
14. Armanios M (2013) Telomeres and age-related disease: how telomere biology informs clinical paradigms. J Clin Invest 123:996–1002
15. Svenson U, Nordfjall K, Stegmayr B, Manjer J, Nilsson P, Tavelin B, Henriksson R, Lenner P, Roos G (2008) Breast cancer survival is associated with telomere length in peripheral blood cells. Cancer Res 68:3618–3623
16. Li H, Engström K, Vahter M, Broberg K (2014) Arsenic exposure through drinking water is associated with longer telomeres in peripheral blood. Chem Res Toxicol 25:2333–2339
17. Richards JB, Valdes AM, Gardner JP, Kato BS, Siva A, Kimura M, Lu X, Brown MJ, Aviv A, Spector TD (2008) Homocysteine levels and leukocyte telomere length. Atherosclerosis 200:271–277
18. Paul L, Cattaneo M, D'Angelo A, Sampietro F, Fermo I, Razzari C, Fontana G, Eugene N, Jacques PF, Selhub J (2009) Telomere length in peripheral blood mononuclear cells is associated with folate status in men. J Nutr 139:1273–1278
19. Liu JJ, Prescott J, Giovannucci E, Hankinson SE, Rosner B, De Vivo I (2013) One-carbon metabolism factors and leukocyte telomere length. Am J Clin Nutr 97:794–799
20. Paul L, Jacques PF, Aviv A, Vasan RS, D'Agostino RB, Levy D, Selhub J (2014) High plasma folate is negatively associated with leukocyte telomere length in the Framingham Offspring cohort. Eur J Nutr 54:235–241
21. Herrmann W, Kirsch SH, Kruse V, Eckert R, Graber S, Geisel J, Obeid R (2013) One year B and D vitamins supplementation improves metabolic bone markers. Clin Chem Lab Med 51:639–647
22. Hubner U, Geisel J, Kirsch SH, Kruse V, Bodis M, Klein C, Herrmann W, Obeid R (2013) Effect of 1 year B and D vitamin supplementation on LINE-1 repetitive element methylation in older subjects. Clin Chem Lab Med 51:649–655
23. Geisel J, Schorr H, Heine GH, Bodis M, Hubner U, Knapp JP, Herrmann W (2007) Decreased p66Shc promoter methylation in patients with end-stage renal disease. Clin Chem Lab Med 45:1764–1770
24. Cawthon RM (2002) Telomere measurement by quantitative PCR. Nucleic Acids Res 30:2–6
25. Werner C, Fürster T, Widmann T, Pöss J, Roggia C, Hanhoun M, Scharhag J, Büchner N, Meyer T, Kindermann W, Haendeler J, Böhm M, Laufs U (2009) Physical exercise prevents cellular senescence in circulating leukocytes and in the vessel wall. Circulation 120:2438–2447
26. Kirsch SH, Herrmann W, Kruse V, Eckert R, Gräber S, Geisel J, Obeid R (2014) One year B-vitamins increases serum and whole blood forms and lowers plasma homocysteine in older Germans. Clin Chem Lab Med 53:445–452
27. Kirsch SH, Knapp JP, Herrmann W, Obeid R (2010) Quantification of key folate forms in serum using stable-isotope dilution ultra performance liquid chromatography-tandem mass

spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 878:68–75
28. Stabler SP, Marcell PD, Podell ER, Allen RH (1987) Quantitation of total homocysteine, total cysteine, and methionine in normal serum and urine using capillary gas chromatography-mass spectrometry. Anal Biochem 162:185–196
29. Kirsch SH, Knapp JP, Geisel J, Herrmann W, Obeid R (2009) Simultaneous quantification of S-adenosyl methionine and S-adenosyl homocysteine in human plasma by stable-isotope dilution ultra performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 877:3865–3870
30. Müezzinler A, Zaineddin AK, Brenner H (2013) A systematic review of leukocyte telomere length and age in adults. Ageing Res Rev 12:509–519
31. Weischer M, Bojesen SE, Nordestgaard BG (2014) Telomere shortening unrelated to smoking, body weight, physical activity, and alcohol intake: 4576 general population individuals with repeat measurements 10 years apart. PLoS Genet 10:1–11
32. Ehrlenbach S, Willeit P, Kiechl S, Willeit J, Reindl M, Schanda K, Kronenberg F, Brandstatter A (2009) Influences on the reduction of relative telomere length over 10 years in the population-based Bruneck study: introduction of a well-controlled high-throughput assay. Int J Epidemiol 38:1725–1734
33. Epel ES, Merkin SS, Cawthon RM, Blackburn EH, Adler NE, Pletcher MJ, Seeman TE (2008) The rate of leukocyte telomere shortening predicts mortality from cardiovascular disease in elderly men. Aging 4:88
34. Nordfjall K, Svenson U, Norrback KF, Adolfsson R, Lenner P, Roos G (2009) The individual blood cell telomere attrition rate is telomere length dependent. PLoS Genet 5:e1000375
35. Aviv A, Chen W, Gardner JP, Kimura M, Brimacombe M, Cao X, Srinivasan SR, Berenson GS (2009) Leukocyte telomere dynamics: longitudinal findings among young adults in the Bogalusa Heart Study. Am J Epidemiol 169:323–329
36. Svenson U, Nordfjall K, Baird D, Roger L, Osterman P, Hellenius ML, Roos G (2011) Blood cell telomere length is a dynamic feature. PLoS One 6:e21485
37. Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, Blasco MA (2006) DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 8:416–424
38. Bull CF, Mayrhofer G, O'Callaghan NJ, Au AY, Pickett HA, Low GKM, Zeegers D, Hande MP, Fenech MF (2013) Folate deficiency induces dysfunctional long and short telomeres; both states are associated with hypomethylation and DNA damage in human WIL2-NS cells. Cancer Prev Res (Phila) 7:128–138
39. Wong JY, De Vivo I, Lin X, Grashow R, Cavallari J, Christiani DC (2014) The association between global DNA methylation and telomere length in a longitudinal study of boilermarkers. Genet Epidemiol 38:254–265
40. Li H, Hilmarsen HT, Hossain MB, Björk J, Hansteen IL, Albin M, Furu Skjelbred C, Broberg K (2013) Telomere length and LINE-1 methylation is associated with chromosomal aberrations in peripheral blood. Genes Chromosomes Cancer 52:1–10
41. Morrish TA, Garcia-Perez JL, Stamato TD, Taccioli GE, Sekiguchi J, Moran JV (2007) Endonuclease-independent LINE-1 retrotransposition at mammalian telomeres. Nature 446:208–212